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PATENT SPECIFICATION

DRAWINGS ATTACHED

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COMPLETE SPECIFICATION

Respiration-Stimulant Substances from Blood

We, SOLCO BASEL A.G., a Swiss Body Corporate, of Freie Strasse 88, Basel, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement: —

This invention relates to a respiration-stimulating active substance for therapeutic purposes and a process for its production.

Blood or serum (i.e. the blood-liquid freed from the blood-corpuscles) has very often been used for therapeutic purposes. Thus for example blood transfusions from one person to another have been carried out and blood from "blood-banks" has been employed in various circumstances. Furthermore blood has been taken from patients themselves, irradiated outside the body, preferably with ultraviolet light, and then reinjected into the patients.

Animal blood has been used for external and internal application in the so-called stimulant therapy. Special applications of blood and its components include serum therapy in infectious diseases, the use of individual blood protein fractions, e.g. the gammaglobulins or antihæmophilic globulins (AHG) in hæmorrhages, the use of Bogomoletz serum and of so-called regenerating sera and the like. In 1891 Haidenhain (Pflugers Arch. 49, 219, 1891) had already discovered vasodilatory and blood pressure-lowering substances in fresh tissue-extracts. Halliburton (Proc. Physiol. Soc. J. Physiol., 24, 7, 1900) found in the blood of paralytics a relatively high quantity of choline and nucleoproteins which originate from the breakdown of the brain tissue and ascribed the paralytic crises to an auto-intoxication with a dilatation of the vessels and lowering of the blood pressure, Popielski (Zbl. Physiol. 16, 505, 1902, Pflugers Arch. 121, 239, 1908, Pflugers Arch. 128, 191, 1909) named the principle active on the blood vessels from the erythrocytes as "vasodilatin" in confirmation of Halliburton's findings.

Freund (Arch. Exper. Path. (All) 86, 266, 1920, Arch. Exper. Path. (All) 88, 39, 1920) from 1920 on injected defibrinated blood in order to examine a vasodilatory and blood pressure-lowering substance, called by him "early toxin". Furthermore he discovered in the blood the so-called "late toxin", a blood pressure-increasing substance. According to Zipf and Wagenfeld (Naunyn Schmiedebergs Arch. 150, 70, 1930) the blood contains toxic substances which affect the blood vessel membranes reducing their permeability and enhance œdema, hyperæmia and the emigration of leukocytes. Also Bergstrasser (Frankfurter Zeitschrift f. Path. 77, No. 2, 1934) found substances when experimenting with blood injections which cause an increase in the number of leukocytes and thereby indicate an increased activity of the bone marrow. The substances observed by the above mentioned workers have in part been identified as adenosine derivatives and acetylcholine and histamine-like substances although their nature is not yet entirely clear. The compounds of the present invention have nothing in common with the vasodilatory blood pressure-lowering or blood-pressure increasing or leucocyte increasing substances mentioned above, whose effect is in part largely due to so-called unspecific protein effects, no attempts having been made for the isolation of active low molecular weight substances. Thus for example the Swiss Patent Specification 90586 describes a process for the production of a preparation from the blood of animals reared on a vitamin-rich diet in which the blood is simply dried.

In addition antibiotically active substances have been demonstrated in the blood e.g. erythrin from erythrocytes (Waksman: "Microbial Antagonists and Antibiotic Substances", N.Y. 1947, p.415) or sanguinin from erythrocytes and hæmoglobin (J. K. Cline, Robert B. Johnson *et al*: Cancer Research 11, 243, 1951; Dorothy M. Whitney, Ludwik Anigstein *et al*, Proc. Soc. Exp. Biol. & Med. 74, 346—350,

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1950). These products are partly acidic and partly neutral in reaction and are fully thermostable. Thus they can resist heating up to 121° C. for 30 minutes without loss of activity and thereby differ from the active principle produced according to the present invention.

Also substances for the stimulation or inhibition of the growth of plant seeds and yeasts are to be found in the blood (see for example F. Venulat & W. Moskwa, Schweiz. med. Wochenschrift 1952, Vol. 3, pp. 35—37). Also Emmerich (Zeitschrift fur Krebsforschung 59 191—208, 1953) occupied himself with the same or similar substances which he found in the blood of patients suffering from various mostly serious diseases. These germ-stimulating or germ-inhibiting products prepared by the processes described have no similarity with the respiration increasing principle prepared by the process of the present invention.

Blood or serum has also been used as starting material for the production of hormones, as in many cases these are present therein in particularly large quantities and can therefore be extracted therefrom more easily and be obtained in purer form, than from the organs, in which they are formed. Thus, for example, gonadotropic hormones can be obtained from the serum of pregnant mares.

In German Patent Specification No. 384,251 there is described a process for obtaining stimulating substances (wound hormones). The blood of animals which have made anæmic is freed from protein and the remaining liquid evaporated to dryness if desired. Here one is concerned with the production of a protein-free serum residue which is intended to be used in therapeutics to achieve an enrichment in red and white blood corpuscles. According to the same process blood-containing organs of the animals so pre-treated are ground and deproteinated after the killing has taken place. The process of preparation (deproteination) which is not fully described is not distinguished by any characteristic and leads to a protein-free serum residue which has no respiration-increasing properties.

Attempts have been made to find further substances with hormone-like character in the blood and in particular to obtain substances with regenerative action on degenerative processes, not from the cells or tissues, in which they are formed, but from the blood, which contains these substances and transports them to the site of their activity.

However it was found that it is extraordinarily difficult to identify substances with regenerative action on degenerative or chronically septic processes in the blood and to isolate them therefrom, since a large number of substances are contained in the blood which possess various functions which mutually counteract or overlap.

We have now found that one can obtain from blood an active substance stimulating respiration if one selects quite definite methods from the many methods known and already applied for the production of substances from blood. In order to find the methods suitable for the purpose desired, i.e. the methods actually leading to the desired active principle, specific known test processes were employed, with the aid of which the presence and the concentration of the active principle can be proved. It may be mentioned that, as is known, the metabolic rate and capacity is increased in regenerative processes, which increase is almost always associated with an increase in respiration.

In the process according to the invention the blood of slaughter-animals is first subjected to dialysis to effect substantially complete deproteination and to effect complete removal of protein particles having a molecular weight in excess of approximately 3000 and the dialysate is concentrated to a dry content of 10—60 mg/ml. This concentrate is then filtered under sterile conditions and filled into ampoules. The product can be used directly for therapeutic purposes.

As stated above, the dialysate is concentrated to a concentration of 10—60 mg of dry substance/ml. Preferably, however the dialysate should be concentrated to a concentration of at least 30 mg. of dry substance/ml. The concentrate obtained according to the invention can, after sterile filtration be used directly in therapeutics if desired.

Any convenient dialysing solvents can be used for the dialysis but water or dilute alcohol are preferably used as solvent. The dialysis is preferably effected in a tube of Cellophane (Registered Trade Mark). Prior to dialysis, it may be convenient to subject the blood to a preliminary deproteination, such as for example by fractional precipitation with alcohols, for example straight or branched chain aliphatic alcohols containing from 1—6 carbon atoms. The fractional precipitation may also be effected by treatment with acid, for example perchloric acid which has the advantage that it can afterwards be easily separated as potassium perchlorate by treatment with alcoholic potassium hydroxide. Trichloracetic acid can also be used but this is less suitable than perchloric acid.

If a more highly purified product with a higher concentration of active substance is however desired the concentrate may be further purified by any suitable procedure. Thus the concentrate may be further purified by precipitation with aliphatic ketones containing from 1—6 carbon atoms or alcohols containing from 1—6 carbon atoms, the active substance being recovered in purified form from the solution in the low molecular aliphatic weight ketone or alcohol, which solution may, after separation from precipitate, be

concentrated *in vacuo* up to effect complete removal of the solvent and is then filtered sterile. Acetone is particularly suitable for use as precipitant.

In another method of purification the concentrate may be extracted with a solvent which is immiscible or only partially miscible with water, e.g. aliphatic alcohols with 3—8 carbon atoms or aliphatic ethers containing from 2—12 carbon atoms. Two phases are thereby obtained an aqueous and a solvent phase, which both contain the active substance in a somewhat comparable concentration. Surprisingly it has been found, that the sum of the quantities of active substance contained in these two phases is greater than that of the untreated concentrate itself. The causes for this phenomenon are not yet clear, but it is assumed, that during the treatment of the concentrate with the immiscible or partially miscible solvent a certain reactivation of the material deactivated by the preceding treatment occurs. Therefore, after the concentration *in vacuo* both phases can be subjected to further working up.

Finally it is also possible to evaporate the concentrate and/or the fractions obtained by the above described purification operations to dryness, to take up the solid product with distilled water and thereupon to subject the solution to sterile filtration and if desired also to freeze-drying under sterile conditions.

The concentrates and purified concentrates obtained according to the invention are in general stable and can be stored for a fairly unlimited period, but if desired preservatives may be added thereto. Suitable preservatives include phenol and cresol.

The blood of slaughter-animals used as starting material according to the invention is preferably that of young slaughter-animals and may be that of such animals which have been put in a condition of altered activity by mechanical or chemical treatment or by irradiation.

It is not necessary to subject the entire blood to the process of the invention; one can separate the blood-cells and the plasma from one another and work them up separately. It may be mentioned that the blood-cells (predominantly erythrocytes) represent as is known as extraordinarily cheap starting material, since no use is found for them in the slaughter houses. The respiration-stimulating active substance provided according to the invention is contained in the blood-cells and plasma in about the same concentration. One can of course also subsequently combine the products obtained from the separate working up of these two blood components.

According to a preferred method of carrying out the process according to the invention the blood may be subjected to a pre-treatment, before dialysis or before the preliminary deproteination preceding dialysis. Thus for example one can haemolize the blood with water or treat the blood with acetone and preferably use the deposit (acetone-dry blood) resulting therefrom alone. Furthermore one can spray-dry it, or defibrinate it by stirring, or partially or completely digest it peptically and/or tryptically.

In the production of acetone-dry blood one obtains an acetone solution containing the principal part of the blood salts, and a precipitate, which shows the principal part of the activity. The peptic or tryptic digestion of the blood may be carried out in known manner by the addition of pepsin at a pH-value of about 2—3 or by the addition of trypsin at a pH-value of about 8—9.

The respiration-stimulating active substances according to the invention are of low molecular weight. With the effects obtained with them, described below, one is apparently not concerned with modified serum effects of unspecific protein effects. The new active substances differ fundamentally from the known active substances conveyed in the blood or obtained therefrom. Thus, in tests in the Warburg-apparatus the new respiration-stimulating active substances produces a respiration increase in tissue homogenates, which could not even remotely be achieved with any substances thus far isolated from blood.

The invention therefore also concerns the extraction product from blood increasing the respiration of tissue homogenates by 100—400%.

The accompanying drawing illustrates the effect of a preparation according to the invention on the respiration of total liver homogenate. The measurement took place in a Warburg apparatus at 37° C. The oxygen consumption was followed manometrically in its temporal course. A rat liver homogenate was made up in Sorensen buffer (pH 7.4) with 1 part of homogenate to 4 of buffer 0.2 ml. of an active substance produced according to the invention was used as addition (with Sorensen buffer adjusted to pH 7.4). Tempering time 10 minutes. Shaking frequency=100/minute. In the drawing the ordinates represent oxygen consumption in cubic millimetres and the abscissae the time in minutes. The curve x-x-x- is of rat liver homogenate alone and the curve o-o-o- that of rat liver homogenate containing 0.2 ml. of the active substance according to the invention.

As is apparent from the drawing, a respiratory increase to about the 4-fold value was achieved after 50 minutes.

The results can be reproduced with the usual biological margin of error of ± 10%.

Hitherto the biological oxidation of adrenaline was ascribed to certain enzymes (e.g. amino oxidases, polyphenol oxidases). It can be shown however, that adrenaline is catalytically oxidized, without the co-operation of an

enzyme, by the low-molecular weight substances obtained according to the invention. This property can therefore be used to test the activity of the substances according to the invention.

The measurement is effected according to the already above-described manometric technique of Warburg, in agitator vessels with 0.4 ml. of normal NaOH-solution per manometer, 0.2—0.4 ml of the principle according to the invention and 0.6 ml of an adrenaline bitartrate solution containing 20 mg of adrenaline bitartrate per 100 mls of solution in the reaction chamber at 38° C., with a shaking frequency of 80—100/min. After passing oxygen through the manometer system for 10 min. followed by a temperature stabilizing period of 10 min. the oxygen consumption is read every 10—20 minutes for 2 hours. An example of the results obtainable is as follows:—

	Oxygen consumption in mm³ after	
	1 hour	2 hours
Active substance alone	7.1	9.8
Adrenaline solution alone	1.4	3.9
Adrenaline solution + active substance	134.2	172.8

The oxygen consumption is proportional to the activity of the substances according to the invention.

In order that the invention may be more fully understood, the following examples are given by way of illustration only:—

EXAMPLE 1.

2 litres of blood from young calves are mixed with 6 litres of acetone with stirring. The resulting precipitate is removed from the clear acetone solution and again stirred with 2 litres of acetone. The operation is then again repeated with 2 litres of a 1:1 acetone-ether-mixture, the precipitate sharply centrifuged off and dried in air. Thus one obtains about 355 g. of an acetone-dry blood, which one can store for several months without loss of activity when kept dry and cool. For the further working up, the dry blood is stirred with 4—5 parts of distilled water and the suspension thus obtained dialysed at approximately 2° C. against the same quantity of distilled water using a Cellophane (Registered Trade Mark) tube. The dialysis operation is repeated until substantially no further quantities of nitrogen containing material migrate into the outer dialysate. The combined outer dialysates are concentrated *in vacuo* to 200 ml at a temperature of not over 22—25° C. The dry weight of this solution amounts to about 40 mg/ml. The solution after sterile filtration and addition of a suitable preservative, e.g. phenol or cresol is directly suitable for therapeutic application. The solution contains in addition to decomposition products of the nucleic acids, amino acids and oligopeptides, which can be characterized according to known paper chromatographic or paper electrophoretic methods. The solution contains a high proportion of the respiration stimulating principle.

EXAMPLE 2.

To calves blood carefully dried in a spray tower are added 5 parts of distilled water, and the resulting mixture worked up in the same manner as acetone-dry blood according to Example 1. The resulting end-product corresponds to that produced according to Example 1 with the exception that it shows a higher salt concentration.

EXAMPLE 3.

1 litre of calves blood freshly taken after slaughter is mixed with 1 litre of distilled water, whereupon haemolysis sets in. The solution is freed of fibrin residues by rough filtration and thereupon dialyzed from a Cellophane (Registered Trade Mark) tube against 2 litres of distilled water. The outer dialysate is changed 4—5 times and the combined outer dialysates are further worked up, as described in Example 1. One obtains approximately 300 ml of concentrate with 40 mg of dry substance/ml. The pH-value of this solution lies at about 8.5 and for the therapeutic application is preferably adjusted with hydrochloric acid to pH 7.

EXAMPLE 4.

2 litres of the fresh calves blood haemolysed according to Example 3 are adjusted to pH 8 and partially digested with 0.2 g. of highly purified trypsin for 24 hours at 37° C. After adjusting to pH 7 a quantitative acetone precipitation is carried out as in Example 1 with 6 litres of acetone. The deposit is stirred again with 2 litres of acetone, then with 2 litres of acetone-ether 1:1, sharply centrifuged off, and dried. The yield amounts to 300—320 g. of dry product, which is further worked up according to the dialysis methods described in the foregoing examples.

EXAMPLE 5.

To 500 ml. of fresh calves blood, defibrinated by stirring, a total of 1000 ml of 96% alcohol are slowly added with stirring within 1 hour at +2° C. The stirring is continued for about 5 hours and the alcoholic solution is thereupon separated from the residue by centrifugation. The alcoholic solution is filtered and concentrated *in vacuo* to 100 ml of alco-

hol-free concentrate at a maximum temperature of 22° C. The pH-value is adjusted to 7. The solution is then slowly brought to a concentration of 80% by weight of alcohol in the cold with stirring by the addition of alcohol (about 400 ml of alcohol are necessary herefor) and left to stand 24 hours at 0° C. The protein deposit which thereby separates contains practically no respiration-stimulating active substances and can be rejected. The alcoholic solution is again concentrated *in vacuo* to 100 ml up to freedom from alcohol and filtered sterile. This solution contains 15 mg of dry substance/ml and possesses a lowering of the freezing point of 1.18° C. It is then subjected to dialysis as described in Example 1.

It is directly suitable for therapeutic purposes.

EXAMPLE 6.

200 ml of fresh, defibrinated calves blood are haemolysed by the addition of 200 ml of distilled water and thereupon mixed dropwise in the cold with approximately 18 ml of 60% perchloric acid with stirring. The deproteination of the blood is complete, when the perchloric acid-concentration is 0.4—0.6 N. Thereupon one stirs for 1 more hour in the cold, the resulting protein deposit is centrifuged off, and washed with 0.4 N perchloric acid and the combined solutions were filtered clear. The solution is mixed in the cold with about 40 ml of 5N KOH up to reaching a pH-value of 7.2—7.3 and the precipitation of the potassium perchlorate is completed by the addition of about 30% by volume of absolute alcohol. One allows it to stand 24 hours at 0° C., centrifugates or filters the solution clear and evaporates off the alcohol *in vacuo*. One obtains 175 ml of the protein-free active substance solution with a dry weight of 17.4 mg/ml and lowering of the freezing point of 1.6° C. which solution is then subjected to analysis as described in Example 1.

EXAMPLE 7.

The plasma or the erythrocyte-sediment obtained by centrifugation can also be worked up according to the working method described in Examples 1—6, usefully after haemolysis. One obtains products stimulating respiration with both starting materials. The best effect is however obtained, if these products are again combined.

EXAMPLE 8.

An active substance solution produced according to the working method of Examples 1—6 is adjusted to pH 5.5 and repeatedly extracted with N-butanol. Thus, for example 100 ml of extract may be extracted 8 successive times with 20 ml. lots of water-saturated butanol. The aqueous and the butanol extract are brought to dryness *in vacuo* at a temperature of not over 20° C. and each taken up with 50 ml of distilled water. According to this process about 15—20% of the dry substance pass from the aqueous phase into the butanol extract. Both fractions are active in the Warburg-test.

EXAMPLE 9.

100 ml. of an extract produced according to the working method of Examples 1—6 are slowly mixed with stirring at pH 7 with 500 ml of acetone. The resulting deposit is centrifuged off, freed from acetone and again taken up in 100 ml of distilled water. This solution contains only a slight activity. The acetone solution evaporated to dryness *in vacuo* is again taken up with 100 ml of distilled water and filtered sterile. It contains the principal portion of the oxygen-activating substances.

WHAT WE CLAIM IS:—

1. A process for the preparation of a respiration-stimulating active substance for therapeutic use which comprises subjecting the blood of slaughter-animals to dialysis to effect substantially complete deproteination and to effect complete removal of protein particles having a molecular weight in excess of approximately 3000 therefrom and concentrating the dialysate thereby obtained to a dry content of 10—60 mg/ml.

2. A process as claimed in Claim 1 in which the dialysate is freed from dialysing solvents prior to concentration thereof.

3. A process as claimed in either Claim 1 or Claim 2 in which the concentrated dialysate is filtered under sterile conditions and filled into ampoules.

4. A process as claimed in any of Claims 1—3 in which water is used for the dialysis.

5. A process as claimed in any of Claims 1—3 in which dilute alcohol is used for dialysis.

6. A process as claimed in any of the preceding claims in which a preliminary deproteination is effected prior to dialysis.

7. A process as claimed in Claim 6 in which the preliminary deproteination is effected by fractional precipitation with alcohols.

8. A process as claimed in Claim 7 in which one or more straight or branched chain aliphatic alcohols containing from 1—6 carbon atoms are used for the precipitation.

9. A process as claimed in Claim 6 in which the preliminary deproteination is effected by fractional precipitation with acid.

10. A process as claimed in Claim 9 in which perchloric acid is used as acid.

11. A process as claimed in any of Claims 6—10 in which the residue obtained after dialysis freed of substances used for the preliminary deproteination is concentrated to a dry substance content of at least 30 mg/ml.

12. A process as claimed in any of Claims 1—11 in which the concentrated dialysate is purified by precipitation with aliphatic ketones containing from 1—6 carbon atoms or ali-

phatic alcohols containing from 1—6 carbon atoms.

13. A process as claimed in Claim 12 in which acetone is used aliphatic ketone.

14. A process as claimed in any of the preceding claims in which the concentrated dialysate is further purified by extraction with a water-immiscible or partly water-miscible solvent and both resulting phases further worked up.

15. A process as claimed in Claim 14 in which aliphatic alcohols with 3—8 carbon atoms or aliphatic ethers with 2—12 carbon atoms are used as solvent.

16. A process as claimed in any of Claims 3—15 in which the crude or purified concentrated dialysate is evaporated to dryness, taken up with distilled water and filtered under sterile conditions.

17. A process as claimed in Claim 16 in which the sterile filtrate is freeze dried under sterile conditions.

18. A process as claimed in any of the preceding claims in which preserving agents are added to the final product.

19. A process as claimed in Claim 18 in which phenol and cresol are used as preserving agents.

20. A process as claimed in any of the preceding claims in which the blood of young slaughter animals is used as starting material.

21. A process as claimed in any of Claims 1—20 in which there is used as starting material the blood of slaughter animals which have been subjected to mechanical or chemical treatment or treatment with radiation to cause therein a state of altered activity.

22. A process as claimed in any of the preceding claims in which the cells and plasma of the blood are separated and both deproteinated by dialysis.

23. A process as claimed in any of Claims 1—22 in which the blood is haemolysed by treatment with water before dialysis.

24. A process as claimed in any of Claims 1—22 in which the blood is treated with acetone and the precipitate deproteinated by dialysis.

25. A process as claimed in any of Claims 1—22 in which the blood is spray-dried before deproteination.

26. A process as claimed in any of Claims 1—22 in which before deproteination by dialysis the blood is defibrinated by stirring.

27. A process as claimed in any of Claims 1—22 in which before deproteination by dialysis the blood is subjected to partial or complete peptic and/or tryptic digestion.

28. A process for the preparation of a respiration stimulating active substance substantially as herein described.

29. A process for the preparation of a respiration-stimulating active substance substantially as herein described with reference to the examples.

30. A respiration-stimulating active substance whenever prepared by a process as claimed in any of Claims 1—29.

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824,375 COMPLETE SPECIFICATION
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